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Hazard/Risk Assessment

TIME-DEPENDENT LETHAL BODY RESIDUES FOR THE TOXICITY OF PENTACHLOROBENZENE TO *HYALELLA AZTECA*

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Abstract—The study examined the temporal response of *Hyalella azteca* to pentachlorobenzene (PCBZ) in water-only exposures. Toxicity was evaluated by calculating the body residue of PCBZ associated with survival. The concentration of PCBZ in the tissues of *H. azteca* associated with 50% mortality decreased from 3 to 0.5 μmol/g over the temporal range of 1 to 28 d, respectively. No significant difference was observed in the body residue calculated for 50% mortality when the value was determined using live or dead organisms. Metabolism of PCBZ was not responsible for the temporal response because no detectable PCBZ biotransformation occurred over an exposure period of 10 d. A damage assessment model was used to evaluate the impact and repair of damage by PCBZ on *H. azteca*. The toxicokinetics were determined so that the temporal toxicity data could be fit to a damage assessment model. The half-life calculated for the elimination of PCBZ averaged approximately 49 h, while the value determined for the half-life of damage repair from the damage assessment model was 33 h.

Keywords—Lethal body residue *Hyalella azteca* Pentachlorobenzene Toxicokinetics Temporal trends

INTRODUCTION

The relationship between the duration of exposure and the dose required to produce a toxic response is well recognized in the toxicology literature. With the advent of the use of body residue as the dose metric, it becomes possible to focus on the internal dose required to produce toxicity in aquatic organisms. Thus, the influence of the toxicokinetic and toxicodynamic factors that influence the response of the organism can be examined. Toxicants that interact strongly with the receptor site (organophosphates with acetylcholinesterase enzyme) were found to produce an essentially irreversible interaction that continued to produce damage yielding a cumulative toxic response [1,2]. The toxicity results from these studies support a model that integrates the exposure duration and suggests that a lower contaminant body residue is required for mortality with longer exposures. These irreversibly bound compounds essentially result in a buildup of damage with increased duration of exposure. Based on the concept that the effect is the result of an accumulation of damage in the organism, models using integrated exposure would be valid not only for compounds where the receptor is irreversibly bound but also where no irreversible binding occurs but the receptor is continuously occupied (constant exposure) and where an irreversible buildup of damage occurs regardless of whether the receptor is occupied (no damage repair).

Laboratory systems often mimic the constant exposure condition such that the receptor would appear to be continuously occupied after steady state has been established. As a result, the toxicant concentration in the tissue of an organism required to produce mortality has been shown to be time dependent for organic compounds acting through a nonspecific mechanism (narcosis). The earliest of these studies with fish suggested that the toxicity of halobenzenes exhibited a linear relationship with the log transformation of time [3,4]. More recent efforts have shown that the time dependence of the toxic response is driven by the accumulation of damage within the organism, and the temporal relationship between body residue and mortality is more complex than a simple logarithmic transformed time relationship [5,6]. The time-dependent relationship between exposure and mortality suggests that organic compounds that act through a nonspecific mechanism can continue to produce damage after the external exposure terminates. This continued damage likely occurs because reversibility of the interaction with the receptor is not instantaneous, and the process for repair requires a finite duration in addition to the elimination process. Thus, damage can accumulate if the exposure is continuous and results in reduced body residues that produce the same cumulative damage that would result in equivalent mortality to that from higher doses with shorter exposures [6]. The studies that established this time-dependent toxicity relationship used selected polycyclic aromatic hydrocarbons (PAH) as the toxicants. Since PAHs can be metabolized, a potential exists that the observation of a time-dependent reduction in body residue to produce mortality could be due to the formation of a toxic metabolite(s), although the metabolites of PAH appear to be less toxic in *Hyalella azteca* than the parent PAH [5]. Thus, a need exists to demonstrate that this time dependence also occurs for compounds that cannot be readily metabolized.

The toxicity of contaminants based on body residue has been determined using several methods. When initially proposed, the body residue was determined as a calculation based on first-order toxicokinetics and the measured concentration for 50% mortality (LC50) to produce a critical body residue

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Table 1. Time-weighted average exposure concentrations for *Hyalella azteca* exposed to pentachlorobenzene in water

Dates and duration of tests	Time-weighted average water concentrations (μmol/L)
April 23–May 21, 2001 (28 d)	0.019, 0.036, 0.078, 0.12, 0.20
June 4, 2001–June 14, 2001 (10 d)	0.32, 0.66, 1.56, 1.93, 2.75, 4.57
July 2, 2001–July 12, 2001 (10 d)	0.22, 0.47, 0.57, 0.79, 1.01
October 29, 2001–November 2, 2001 (4 d)	0.73, 1.02, 1.32, 1.70, 1.90
November 19, 2001–December 14, 2001 (25 d)	0.42, 0.57, 0.78, 0.99, 1.14

(CBR) for 50% mortality [7]. More recent work has determined the body residue associated with 50% mortality in exposed organisms using two methods. The first method used the chemical concentration in live organisms measured at specific times and the observed response to derive a body residue response relationship termed a lethal residue concentration for 50% mortality (LR50; e.g., [8]). Others have determined the average concentration in dead organisms and tied it to the time to 50% mortality in the sample population (LT50) as the internal lethal concentration (ILC50) [3] or mean lethal residue (MLR50) [5]. Whether the LR50 calculations were calculated using dead organisms or live organisms for body residues as the representation of exposure, the estimated LR50 values for a C-12 linear alkylbenzene sulfonate were the found to be the same in the midge *Chironomus riparius* [9]. Thus, it is expected that these two methods used to determine the body residue required to produce 50% mortality should yield equivalent values.

The main objective of the current study was to determine the time-dependent toxicity of pentachlorobenzene and compare the results to that observed for PAH with *H. azteca*. The second objective was to demonstrate the damage assessment model [6] for a compound that is not biotransformed, thus supporting the hypothesis that damage for nonpolar narcotics is not rapidly reversible. Pentachlorobenzene was selected for the study because it has a limited potential for biotransformation and would be readily bioaccumulated by aquatic organisms. The third objective was to demonstrate that the methods using body residues as the dose metric, MLR50 or LR50, produced equivalent toxicity estimates.

MATERIALS AND METHODS

Chemicals

The ^{14}C-pentachlorobenzene (PCBZ, log K_{ow} 5.18 [10]) was purchased from Sigma Chemical (10 mCi/mmol; St. Louis, MO, USA), and nonlabeled compound was purchased from Chem Services (West Chester, PA, USA). The water solubility of PCBZ has been reported to range from 0.18 to 1.34 mg/L [11]. The vapor pressure for PCBZ in the same review ranged from 0.219 to 1.08 Pa at 25°C [11]. Radiopurity of the pentachlorobenzene was determined before use by a combination of thin-layer chromatography (TLC; hexane:benzene, 95:5, v:v) on silica gel plates and liquid scintillation counting (LSC) on a Packard Tri Carb model 2500 (Packard Instruments, Meriden, CT, USA) by removing the silica gel in sections and counting the associated radioactivity. The amount of radioactivity was determined using the external standard ratio method after subtracting background. Radiopurity was determined to be greater than 98% using the TLC method. Because mass balance is difficult to maintain because of the volatility of the PCBZ, the purity of the radiolabeled compound was also determined via gas chromatography/mass spectrometry using the conditions described below, except that the mass spectrometric detector was run in the mass scanning mode. The only compound found after subtracting the background was PCBZ.

Dosing stock solutions were made by generating a nonlabeled stock in acetone and then adding the appropriate amount of ^{14}C-labeled PCBZ in acetone to create the final dosing stock. New specific activities were determined for each dosing stock solution based on the determination of the concentration of the radioactive material by LSC and the nonlabeled material by gas chromatography/mass spectrometry. The gas chromatography/mass spectrometry determinations were made by diluting the PCBZ dosing stocks in hexane and spiking with a trichlorobiphenyl internal standard (International Union of Pure and Applied Chemistry 30) prior to injection on a Hewlett-Packard 5890 Series II gas chromatograph with a 5971 series mass selective detector (Agilent, Palo Alto, CA, USA) using a DB-5 column with helium as the carrier gas. Data were acquired using the selected ion-monitoring mode. The gas chromatograph conditions were 50°C at the beginning of a sample run and a temperature program ramping at 25°C/min to 150°C, then 6°C/min to 200°C, and finally 15°C/min to 300°C. A multiple-point PCBZ standard calibration curve was used for quantification.

Organisms

Hyalella azteca, age 7 to 10 d old, were purchased from Aquatic Bio Systems (Fort Collins, CO, USA). On arrival, the organisms were acclimated to the test water by replacing 50% of the water they were shipped in with filtered (glass-microfiber filters 934-AH; Whatman, Clifton, NJ, USA) Huron River (Dexter, MI, USA) water collected upstream from the Hudson Mills Metropark. The water characteristics were pH of 8.1 to 8.3, hardness as mg $CaCO_3$/L of 165 to 250, and alkalinity as mg $CaCO_3$/L of 170 to 250 [8].

Experimental design

Several experiments were conducted to characterize the toxicity, kinetics, and biotransformation of PCBZ at different exposure durations (4, 10, and 28 d) as water-only exposures. These exposures were conducted to derive uptake (k_u) and elimination (k_e) rate constants for PCBZ as well as to assess the effects on *H. azteca* survival and growth. In similar experiments, the biotransformation of PCBZ was determined at 4 and 10 d. Toxicity of PCBZ was determined primarily as mortality except in one 28-d test where organism growth was examined as the toxic response. The *H. azteca* were exposed to a range of PCBZ concentrations ranging from 4.8 μg/L (0.019 μmol/L) to 0.92 mg/L (4.57 μmol/L) as the time-weighted average water concentration (Table 1) in 200-ml beakers filled with 150 ml of test solution. Water was dosed with

PCBZ daily using acetone (100 µl/L) as the carrier, and the control received the same acetone concentration as the treatments. In the initial experiment evaluating growth and toxicokinetics, only 75% of the water was changed daily. For all other experiments, complete water exchanges were performed. Exposures employed three replicates per sampling time with two to five sampling times per experiment. Each exposure beaker received 10 *H. azteca*, except for the biotransformation experiment, which began with 25 *H. azteca* at day 0. A 1- to 2-cm^2 square of sterile cotton surgical gauze was placed in the beakers as a substrate, and 0.25 ml of yeast-cerophyl-trout-chow (the recipe yields 1.7–1.9 g/L [12]) was added daily except for the biotransformation experiment, which was given 0.35 ml. The experiments were conducted between 20 and 22°C. Dissolved oxygen was measured periodically throughout the experiments just prior to water exchange. Water samples (2 ml) were collected before and after water exchanges to determine time-dependent water concentrations. During each water exchange, dead (completely immobilized) organisms were removed and weighed, and body residue concentrations were determined by LSC. Sample times depended on the duration of the experiment with shorter studies sampled frequently (hourly to daily), while longer studies were sampled approximately geometrically with the first sample on day 1 or day 2 and the last sample at the end of the exposure. At each sampling point, surviving *H. azteca* were counted and body residue concentrations determined from each of three replicate beakers. The amount of compound in the organism and water samples was determined by LSC. The water samples and organisms were added directly to scintillation cocktail (3a70b, RPI International, Mount Prospect, IL, USA). The organism samples were held at least overnight prior to counting to allow the xylene-based scintillation cocktail to serve as the extracting solvent for the PCBZ [13]. The concentrations were determined based on the amount of radioactivity and the specific activity of the appropriate exposure stock.

The percentage of PCBZ biotransformation to metabolites was determined by using a similar experimental design as the 10-d toxicity experiments. Eight replicates were used with 25 *H. azteca* per replicate. Half the replicates were sampled on day 4, while the other half were sampled on day 10. A subsample of the organisms was analyzed for total body residue analysis, and the remainder of the organisms was kept frozen at −20°C until extraction for TLC analysis. The tissue samples were extracted with 2 ml acetone followed by 1 ml of dichloromethane and 1 ml of diethylether and the extracts combined. The biotransformation was determined twice, and in the second experiment, the quantity of unextractable PCBZ metabolites in the extracted residue was also determined. Following extraction, tissue residue fragments were dispersed in scintillation cocktail and counted for bound activity (unextractable residue). The unextractable residue was not different from background. The sample extracts were combined and dried over anhydrous sodium sulfate. A subsample was taken for LSC to determine the total amount extracted (average extraction efficiency 90 ± 14%, mean ± standard deviation, n = 6). The extract was reduced in volume under a stream of nitrogen and spotted for TLC analysis as was done for the original stock (described previously). The fraction of metabolite was determined as the sum of the tissue-bound residue and the nonparent activity on the TLC plate divided by the total expected activity based on total body residue measurements by LSC. Parent compound was determined as the difference between the expected total and the measured amount of metabolites.

The percentage of total lipid was determined using a spectrophotometric method [14]. For the 28-d test in May 2001 and the 10-d tests in June and July, the lipid content of *H. azteca* was determined at day 0 and at the end of the experiments using the organisms sampled from the control group and from selected treatments where an adequate number of organisms were alive. Lipids were determined in 10 organisms at $t = 0$ for the 28-d test, five organisms at $t = 0$ for the 10-d tests, and in three to five organisms per dose at the end of the tests.

Statistics and modeling

Modeling for both the toxicokinetics and the damage assessment models were performed using Scientist® Version 2.01, (MicroMath, St. Louis, MO, USA). Comparisons of means were performed using Student's t test, and significance was set at $p \leq 0.05$. Error values in the manuscript are given as standard deviation unless otherwise stated.

Toxicity was determined by logit analysis of the response versus mean live organism concentration for each replicate at the specific sampling times to produce LR50 estimates. The mean body residue concentration in dead organisms for each exposure concentration was determined and associated with the LT50 determined through the logit analysis to yield an MLR50 [3]. The MLR50 values were also estimated from the mean body residue concentration in live organisms post-LT50 to find an MLR50.

Toxicokinetics were fit to a one-compartment model:

$$\frac{dCa}{dt} = k_u Cw - k_e Ca \quad (1)$$

where k_u is the uptake clearance (ml/g/h), Cw is the concentration in the water (nmol/ml), k_e is the elimination rate constant (per h), Ca is the concentration in the organism (nmol/g), and t is time (h). The water concentration was initially modeled as time variable to account for the decline that occurred between water renewals. However, the resulting estimates for the toxicokinetic constants were found to be identical when the water was held constant using the time-weighted average water concentration. The frequency of the water exchange (≈24 h) is substantially faster than elimination for the organism ($t_{½}$ ≈ 41 h), which allows for the use of a time-weighted average.

The time-dependent toxicity data were then fit to a damage assessment model that accounts for the toxicokinetics and the toxicodynamics [6]. The model was constructed to account for the dynamic accumulation of the contaminant (Eqn. 1) with a dynamic formation and repair of damage (Eqn. 2) as a result of the amount of accumulated compound. The simplest first approach for the formation of damage is to use a first-order model proportional to organism concentration. Further, the repair was assumed to be proportional to the amount of damage. The result of these two assumptions yields a first-order model for damage accumulation:

$$\frac{dD}{dt} = k_a C_a - k_r D \quad (2)$$

If a critical damage level exists that results in 50% mortality, D_L, then the damage assessment model that accounts for the toxicokinetics and the toxicodynamics is as follows:

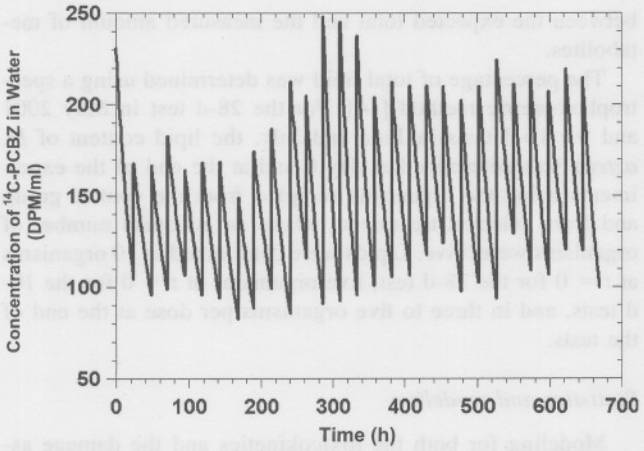

Fig. 1. Variation in water concentration at 0.019 μmol/L pentachlorobenzene (PCBZ) as the time-weighted average. Specific activity = 8.05×10^6 disintegrations per minute (DPM)/μmol.

$$LR50(t) = \frac{\frac{D_L}{k_a}}{\frac{1}{(1 - e^{-k_e \cdot t})} \times \left(\frac{e^{-k_r \cdot t} - e^{-k_e \cdot t}}{k_r - k_e} + \frac{1 - e^{-k_r \cdot t}}{k_r} \right)}$$

where D_L (unitless) is the critical level of damage that results in 50% mortality, k_a is the rate constant for damage formation (nmol/g/h), and k_r is the rate constant for damage repair (per h).

RESULTS

Exposure conditions

Contaminant concentrations in the exposure water declined during the period between renewals during the *H. azteca* toxicity experiments. The ^{14}C-PCBZ concentration had an average mean decline of 49.1 ± 8.1% (n = 21; Fig. 1) between water renewals. The period between consecutive renewals varied from 21 to 28 h in the experiments. To account for the variation in water concentration, the time-weighted average water concentrations were calculated to serve as the exposure concentrations for each treatment (Table 1). The justification for the use of the time-weighted average concentration to represent the exposure is demonstrated in the *Toxicokinetics* section. The loss of PCBZ from the exposure beakers is most likely the result of volatilization from the water. Potential sorption to the gauze was not evaluated as a source for exposure or a contribution to declining water concentrations. However, if the compound were bound to the gauze, it would have had a reduced bioavailability. Thus, the water was considered the source of contaminant to the amphipods for the purposes of the toxicokinetics.

The dissolved oxygen (DO) generally did not drop below 5.9 mg/L in the mortality studies where the water was completely exchanged. However, in the 28-d growth study where only 75% of the water was exchanged daily, DO was found to drop substantially after day 10. For example, DO declined to 2.4 ± 0.6 mg/L (n = 24) on the last day of the study, and no difference was observed in the DO among treatments. This apparently occurred because of periphyton growth on the sides of the beakers. No significant impact of reduced DO on survival of the *H. azteca* was observed, as the survival was 90% or greater in all treatments and the control.

Lipid content

Lipid content of organisms in the 28-d experiment was 4.5 ± 1.1% (n = 10) of dry weight at time 0 h and 7.7 ± 2.4% (n = 18) at the end of the 28-d exposure. An increase was observed in lipid content over the course of the study, and no statistically significant difference was seen in the lipid content between the control and the treatments or among the treatments. For the 10-d experiments in June and July 2001, the average percentage lipid content of the *H. azteca* was 6.7 ± 1.7% (n = 26). No difference was seen in lipid content between initial and final sample times.

Biotransformation

Exposures to examine biotransformation were done to confirm the expectation that *H. azteca* would not biotransform the highly chlorinated PCBZ. The total amount of radioactivity recovered from the TLC plate was 37.5 ± 12.8% (n = 6) of the total expected based on whole-animal LSC analysis. Based on the known extraction efficiency of this method, the loss was most likely the result of volatilization during the processing of the samples and the elution on TLC. It was assumed that all volatilized material was parent compound because metabolites such as pentachlorophenol, a potential phase 1 biotransformation product, has a vapor pressure that is about 2% of that for PCBZ [15]. While a large portion of the PCBZ had volatilized, only 0.34 ± 1.2% (n = 6) of the total radioactive material on the TLC plate was not parent compound, and no unextracted residue was detected. Based on the amount of activity initially spotted on the plates, the limit of detection for any biotransformed product (three times background) would have been less than 0.5%. Because most of the volatilized compound was parent PCBZ and only a small fraction of the radioactivity remaining on the TLC plate was not parent compound and since no unextractable metabolites were detected, it can be assumed that the extent of biotransformation was negligible.

Toxicokinetics

The toxicokinetics were originally fit using the first-order model assuming no biotransformation but with a variable water concentration. The assumption of no biotransformation was found to be valid based on the data presented previously. Because of the variable water concentration, the model had to be integrated numerically and used a fourth-order Runge–Kutta algorithm (Fig. 2). While the concentration in the organism varied as the concentration in the exposure solution declined on a daily basis between water exchanges, the modeled decline in body residues on a daily basis was small relative to the measured variability in body residue. The decline from the daily peak in the body residue (Fig. 2) was relatively small because of the slow elimination kinetics for the PCBZ (Table 2). When the data were fit using the time-weighted average water concentration, the observed toxicokinetics produced similar estimates for the rate coefficients: 44.8 ± 15.5 ml/g/h (n = 60) versus 44.8 ± 13.15 ml/g/h for k_u and 0.023 ± 0.008/h versus 0.023 ± 0.007/h for k_e for the time-variable and time-weighted average water concentrations at the 0.019-nmol/ml exposure, respectively. Because the estimates were essentially identical, the toxicokinetic values were estimated for the rest of the data using the time-weighted average water concentration (Table 2).

Despite sampling the *H. azteca* temporally, it was not al-

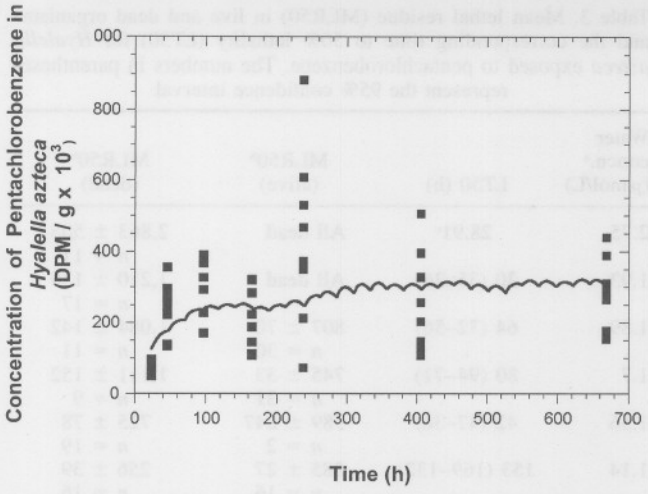

Fig. 2. Bioaccumulation of pentachlorobenzene (PCBZ) by *Hyalella azteca* fit to a one-compartment model (Eqn. 1) exposed at 0.019 μmol/L PCBZ with time-variable water concentration. Specific activity = 8.05×10^6 disintegrations per minute (DPM)/μmol.

ways possible to obtain an estimate of the toxicokinetics. This was primarily because in the longer experiments, the time course sampling occurred after the organisms had approached steady state. Since the bioconcentration factor (BCF) is the ratio of k_u divided by k_e, it is possible to obtain some estimate of the uptake clearance assuming that the elimination does not vary with dose. This was accomplished by multiplying the measured BCF by the average value for k_e determined from the experiments where complete toxicokinetics could be determined (0.014 ± 0.008, n = 11). The result is that a decrease in the uptake clearance is apparent as the concentration increases up to a concentration of about 0.5 nmol/ml, after which

Table 2. Pentachlorobenzene toxicokinetics and bioconcentration factor (BCF) in *Hyalella azteca* determined using the time-weighted average water concentration as the exposure concentration for all exposures where the uptake rate coefficient (k_u) and the elimination rate constant (k_e) could be estimated

Concn. (μmol/L)	k_u[a] (ml/g/h)	k_e[a] (h)	BCF measured[a]
0.019	44.8 ± 13.1	0.023 ± 0.007	1,913 ± 222 n = 30
0.036	57.1 ± 10.3	0.022 ± 0.005	1,452 ± 193 n = 29
0.078	43.6 ± 15.3	0.023 ± 0.009	1,874 ± 221 n = 30
0.12	40.7 ± 16.0	0.019 ± 0.009	2,164 ± 314 n = 28
0.20	35.3 ± 11.0	0.016 ± 0.006	2,258 ± 264 n = 27
0.32	20.4 ± 5.3	0.012 ± 0.005	1,139 ± 118 n = 19
0.42	11.4 ± 1.9	0.002 ± 0.001	2,143 ± 230 n = 26
0.47	8.98 ± 2.6	0.005 ± 0.004	871 ± 78 n = 30
0.56	11.6 ± 4.0	0.014 ± 0.007	666 ± 59 n = 30
0.57	6.1 ± 1.2	0.005 ± 0.002	780 ± 64 n = 21
0.78	8.8 ± 3.2	0.013 ± 0.006	577 ± 65 n = 20

[a] Mean ± standard error.

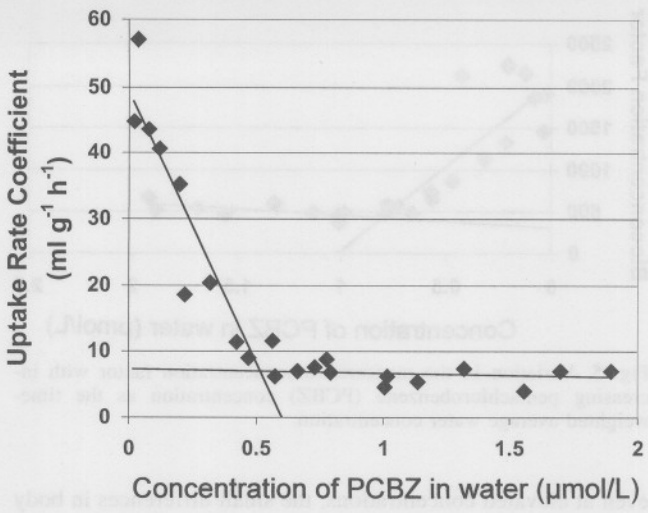

Fig. 3. Variation in the uptake rate coefficient (k_u, ml/g/h) with increasing pentachlorobenzene (PCBZ) concentration as the time-weighted average water concentration.

the uptake clearance stabilizes at an average value of 8.1 ± 1.2 ml/g/h (n = 15; Fig. 3).

The elimination rate constant was variable and declined with increasing exposure concentration (Fig. 4), and the corresponding half-life for elimination ranged from 21 to 346 h. However, one outlier in the elimination data exhibited a high elimination rate constant (0.033/h) at a high exposure concentration of 0.989 nmol/ml.

The bioconcentration factor showed a similar pattern to that of the uptake clearance, with the BCF becoming constant at about 500, where the PCBZ concentration was about 0.7 nmol/ml (Fig. 5). Also, the BCF determined from the concentrations in the dead organisms were essentially the same as those determined from live organisms with a slope of 1.31 ± 0.4 (r^2 = 0.45; Fig. 6). This is critical since the two measures of mortality, LR50 and MLR50, use body residues measured in live and dead organisms, respectively. At the higher water concentrations, the concentrations in the live organisms were somewhat elevated compared to the dead organisms. This difference may reflect the differential sensitivity of the dead organisms. Regression analysis demonstrated that the slope of the line is not statistically different from one, and therefore,

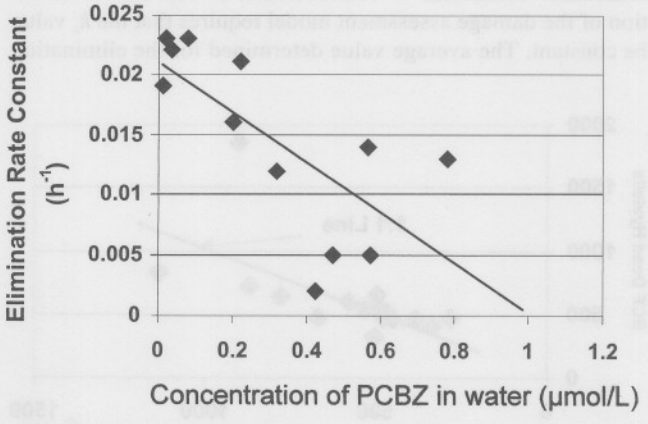

Fig. 4. Variation in the elimination rate constant (k_e, per h) with increasing pentachlorobenzene (PCBZ) concentration as the time-weighted average water concentration. The k_e value of 0.033/h at 0.989 nmol/ml was omitted from the figure as an outlier.

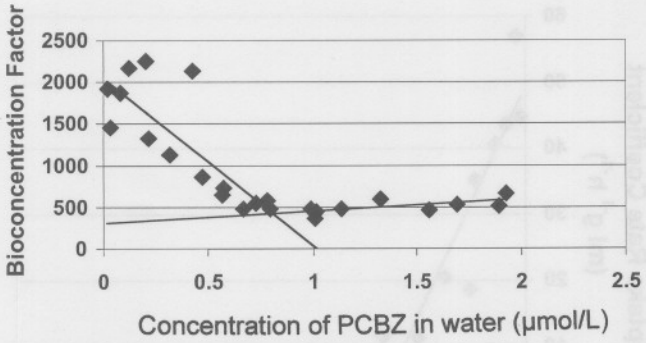

Fig. 5. Variation in the measured bioconcentration factor with increasing pentachlorobenzene (PCBZ) concentration as the time-weighted average water concentration.

even at elevated concentrations, the small differences in body burden would not affect the measures of mortality.

Toxicity: Mortality and growth

The mortality data were determined for varying lengths of exposure to PCBZ. This required several experiments to cover the range of concentrations required to produce 50% mortality. The only data used to evaluate the effect of exposure time on toxicity were those that exhibited well-defined 95% confidence limits using the logistic regression model (Tables 3 and 4). The values exhibited a decline in the required internal concentration required to produce mortality with increasing duration of exposure (Fig. 7). Determining the body residue for 50% mortality by either the LR50 method or the MLR50 method did not result in any difference in the estimate for values determined at the same time (Fig. 7). Despite the nearly equal BCF values between live and dead organisms, some difference was observed in the estimated MLR50 values, with those for the live organisms generally lower than those for the dead (Table 3). However, the ratio of $MLR50_{dead}$ divided by $MLR50_{live}$ was 1.06 ± 0.33 (n = 8; Table 3). Thus, the difference is small and does not change the overall temporal trend (Fig. 7). For the longer-term exposures (>150 h), the concentration required for 50% mortality appears to essentially reach an asymptote, and the values are statistically indistinguishable.

The damage assessment model described the variability in the data, which included a combination of all the estimates for body residue required to yield 50% mortality, (Fig. 7) with a reasonable coefficient of determination (0.48). Parameterization of the damage assessment model requires that the k_e value be constant. The average value determined for the elimination

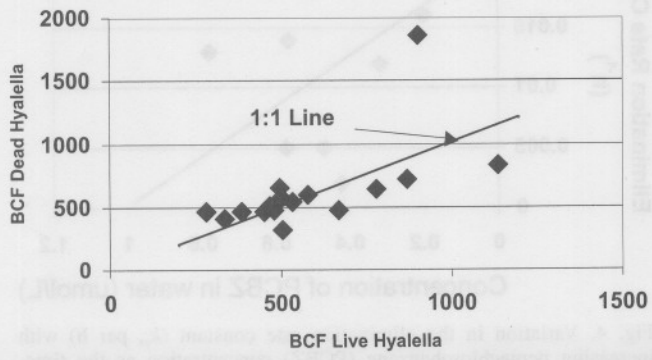

Fig. 6. Relationship of the bioconcentration factor (BCF) measured in live versus dead *Hyalella azteca*.

Table 3. Mean lethal residue (MLR50) in live and dead organisms and the corresponding time to 50% lethality (LT50) for *Hyalella azteca* exposed to pentachlorobenzene. The numbers in parentheses represent the 95% confidence interval

Water concn.[a] (µmol/L)	LT50 (h)	MLR50[b] (alive)	MLR50[b] (dead)
2.75	28.91[c]	All dead	2,863 ± 503 n = 11
1.93	30 (35–26)	All dead	1,210 ± 130 n = 17
1.89	64 (72–56)	807 ± 70 n = 30	1,084 ± 142 n = 11
1.7	80 (94–71)	745 ± 33 n = 31	1,111 ± 152 n = 9
1.56	42 (47–36)	589 ± 247 n = 2	725 ± 78 n = 19
1.14	153 (169–132)	385 ± 27 n = 16	256 ± 39 n = 16
1.01	121 (152–94)	348 ± 30 n = 23	413 ± 32 n = 16
0.99	198 (212–179)	424 ± 29 n = 17	499 ± 36 n = 12
0.78	267 (308–232)	460 ± 39 n = 30	376 ± 55 n = 4
0.57	388 (493–322)	467 ± 55 n = 29	271 ± 84 n = 3

[a] Time weighted average water concentration.
[b] Mean ± standard error of the mean lethal residues for 50% mortality (MLR50).
[c] Value is an estimate since the 95% confidence interval could not be calculated for this experiment.

rate constant (0.014/h) was used. Selecting the average value was based on the fact that the LR50 values, whether determined through measured body residue or calculated from the LC50 using the measured BCF value, were determined across the whole range of concentrations explored, and thus the average value should be applicable as the representative k_e value. The potential impact of choosing the average value was investigated by examining the impact of using the extreme values for k_e in the model. The ratio of D_L/k_a increased by as much as 10% with the largest measured k_e value (0.033/h) and decreased by 4% with the smallest k_e value (0.005/h). However,

Table 4. Lethal body residue for 50% mortality (LR50) and the lethal concentration for 50% mortality (LC50) for individual sample times. The values in parentheses represent the 95% confidence interval

Time (h)	LR50 (nmol/g)	LC50 (nmol/ml)
48.2	690 (811–594)	
73.9	411 (512–241)	
96.7	568 (675–490)	
96.8	381 (653–276)	
144	807 (10,139–498)	
241	346 (426–213)	
26.5		2.3 (2.5–2.1)
49		1.1 (1.2–0.9)
74		1.9 (2.2–1.7)
96		1.4 (4.0–1.1)
96.7		1.4 (1.6–1.3)
96.9		0.84 (1.0–0.7)
144		1.0 (1.8–0.8)
169		1.4 (3.9–1.1)
240		0.8 (0.9–0.7)
241		0.7 (0.8–0.65)
409		0.5 (0.6–0.45)
600		0.5 (0.7–0.49)

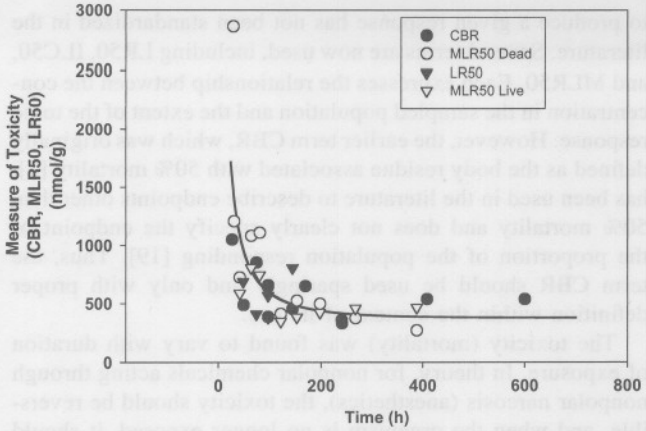

Fig. 7. Time-dependent mortality determined using several methods to estimate the body residue for 50% mortality. The values labeled CBR reflect the calculated body residue from the measured median lethal concentration and the toxicokinetics. The fit of the damage assessment model to the estimates for the body residue required for 50% mortality. D_L/k_a = 18,077 (2,752) nmol·h/g, and k_r = 0.021 (0.007)/h, n = 36. MLR50 = mean lethal residue; LR50 = lethal residue concentration for 50% mortality.

the k_r varied less than 5%, ranging from 0.20 to 0.21 over the range of k_e values.

The model allows prediction of the time-dependent mortality when organisms are exposed under a variety of conditions from short-pulsed exposures to long-term constant exposures. The body residue for 50% mortality measured at short exposures (24–48 h) were in the range of 1 to 3 μmol/g, which suggests that the PCBZ likely acts by nonpolar narcosis (anesthesia). This range of concentrations is consistent with the 2 to 8 μmol/g described for nonpolar narcosis [16]. The longer-term exposures (t > 150 h) required body residues in the range of 0.44 μmol/g and, based on the damage assessment model, indicates a buildup of damage with longer exposures. These time-dependent values for the body residue response of *H. azteca* for short- and long-term exposures are consistent with the concentrations required for acute and chronic mortality in fish respectively [16].

In two longer-term exposures of 10 and 25 d, it was not possible to determine the LR50 values because a dose response on a body residue basis was not clear. However, MLR50 values could still be determined for the higher concentrations. Furthermore, for some of the lower doses, the accumulated body residue was higher than the MLR50, while *H. azteca* exhibited lower mortality. The mechanisms behind these observations are not clear at this time but likely result from the ability of the organism to repair damage at lower exposure concentrations. As a result of these observations, the MLR50 values in these longer exposures should be considered the minimum required to produce 50% mortality. Clearly, this issue requires additional investigation.

The variability of body residue concentrations associated with effects on growth rate (per h) was more variable than mortality as the toxicity endpoint (Fig. 8). The estimated concentration required to produce a 50% reduction in the growth rate was 0.87 μmol/g. Thus, while significant reductions in growth rate occur at lower concentrations than required for 50% mortality, the concentration that reduces growth rate by 50% is similar, within about a factor of two, to the concentrations required to produce 50% mortality with long (>150 h) exposures. The variability was thought to result from the

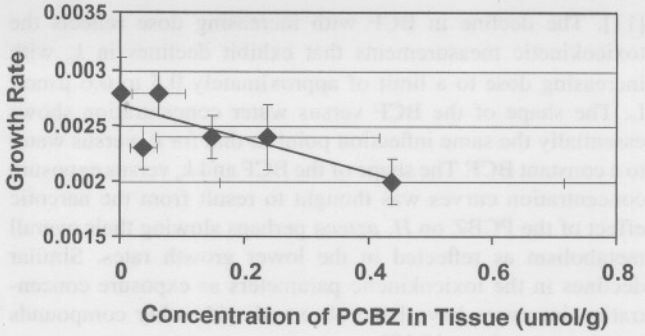

Fig. 8. The growth rate for *Hyalella azteca* measured in a 28-d growth study (showing mean ± standard deviation) declined with increasing pentachlorobenzene (PCBZ) body residue according to the following: growth rate = −0.0015 (PCBZ tissue concentration) + 0.0027, r^2 = 0.63, n = 6.

difficulty in measuring both the weight of individual *H. azteca* and the ability to determine the concentration of PCBZ in individual organisms. However, some of the variability may be a result of differences in the growth rates of individual organisms.

DISCUSSION

The overall purpose of this work was to establish the time-dependent body residue–mortality relationship for use in predicting the toxicity of PCBZ to *H. azteca* under variable exposure conditions. The toxicokinetic parameters must be known to develop the time-dependent mortality relationship for prediction of the expected effect of PCBZ in *H. azteca* using the damage assessment model [6]. Thus, it was important to establish the toxicokinetics under a variety of conditions, such as exposure concentration and exposure duration. These values were determined in concert with an assessment of organism mortality and growth. With longer-term studies, it was not possible to determine the detailed toxicokinetics because the sampling regime was primarily data collected after the organisms had achieved steady state. Furthermore, the elimination rate constant exhibited a trend toward slower elimination with increased dose (Fig. 4). However, the data were quite variable, and the actual extent of decline was not clear. Thus, much of the variability could have been due to the sampling regime that tended to sample organisms later in the kinetic profile, resulting in less certain estimates of the kinetic rate constants.

Both the uptake and elimination rate coefficients and the measured BCF declined with increasing PCBZ exposure concentration (Figs. 3 and 5, respectively). Part of the observed decline in measured BCF may have been due to failure of the amphipods to achieve steady state by the end of some of the exposures, particularly at higher exposure concentrations. However, the shape of the BCF–water concentration relationship was similar to that for k_u, suggesting that k_u was the controlling factor in the BCF calculation. This supports the contention that k_e was perhaps not as variable as suggested in Figure 4. The BCF determined in this work ranged from a low of about 500 to a maximum of 2,200 (Fig. 5). The high values found at the lower exposure concentrations are similar to the low end of the range of BCF values reported in the literature [11]. The low BCF at low exposure concentrations reflects, in part, the low lipid content of *H. azteca* (4–7% on a dry-wt basis). If adjusted for lipid content, the BCF would be similar to that reported on a lipid-weight basis for other organisms

[11]. The decline in BCF with increasing dose reflects the toxicokinetic measurements that exhibit declines in k_u with increasing dose to a limit of approximately 0.5 to 0.6 μmol/L. The shape of the BCF versus water concentration shows essentially the same inflection point as that for k_u versus water to a constant BCF. The shape of the BCF and k_u versus exposure concentration curves was thought to result from the narcotic effect of the PCBZ on *H. azteca* perhaps slowing their overall metabolism as reflected in the lower growth rates. Similar declines in the toxicokinetic parameters as exposure concentration increases have been observed with other compounds in other organisms [8,17].

It was important to demonstrate that the measured concentrations in the dead organisms were essentially the same as those in live organisms. Previous studies examining body residue response relationships have shown that it did not matter whether the concentrations were measured in dead or live organisms [9]. But it was particularly important for this work because the two methods of determining the concentration required to produce 50% mortality, LR50 using live organism concentrations and MLR50 using dead organism concentrations, rely on different subpopulations. In the normal dose–response curve, organisms that are more sensitive should respond at lower internal concentrations than organisms that are more resistant. Thus, a bias might exist between the determinations based on live or dead organisms. The measured BCF values did not show any significant bias between the two groups, suggesting that the two measurements would be equally successful for describing the body residue that is associated with 50% mortality. This is particularly important for calculating a toxicity measure in the general assessment of the impact of bioaccumulated residue. In a regulatory program, the approach would have to be simplified, and the dead organisms, which degrade rapidly in sediment, would not be available for analysis. Therefore, live organisms will be used to have enough biomass for chemical analysis.

Several methods are available in the literature for estimating the body residue that elicits 50% mortality in aquatic organisms. Early work used the BCF and LC50 measurements to estimate the residue concentration for 50% mortality and was called the CBR [7]. This approach was suggested as an approach for improving the interpretation of body residues and bioaccumulation of organic chemicals [16]. Several investigators have measured the body residue in dead organisms to estimate the concentration associated with 50% mortality. The ILC50, calculated from the LT50 and the mean concentration in dead organisms [3,4], was later termed MLR50 [5]. In addition, the concentration in dead organisms was used to calculate LR50 values at specific exposure times along with a comparison to that in live organisms. These experiments were conducted at the same exposure times to demonstrate that either measure provided the same population estimate [9]. Finally, the use of live organisms to calculate LR50 values was developed for estimates at fixed exposure times [8,18]. This work provides the first examination of all three approaches for estimating the body residue to produce 50% mortality (Fig. 7). All the estimates show the same time dependence, and all the estimates cluster together along the same time line (Fig. 7). Thus, for the future, it is clear that it will be possible to determine the body residue–based toxicity measurements using live organisms or calculated from the appropriate kinetic parameters and estimates of the LC50.

The terminology for expressing the body residue required to produce a given response has not been standardized in the literature. Several terms are now used, including LR50, ILC50, and MLR50. Each expresses the relationship between the concentration in the sampled population and the extent of the toxic response. However, the earlier term CBR, which was originally defined as the body residue associated with 50% mortality [7], has been used in the literature to describe endpoints other than 50% mortality and does not clearly specify the endpoint or the proportion of the population responding [19]. Thus, the term CBR should be used sparingly and only with proper definition within the context of its use.

The toxicity (mortality) was found to vary with duration of exposure. In theory, for nonpolar chemicals acting through nonpolar narcosis (anesthetics), the toxicity should be reversible, and when the organism is no longer exposed, it should recover. In the damage assessment model, the recovery depends on both the rate of elimination and the rate of damage repair. Since the elimination rate would include the rate of removal from the membrane, the proposed site of action, the recovery of the damage must include other biological processes that must come into balance after the compound is eliminated. While other approaches, such as the use of constant area under the curve [1] or constant target occupation models [2], would provide a good description of the time dependence for the toxicity, these models require the chemical to be irreversibly bound to the receptor site. The damage assessment model [6] does not have this requirement and actually estimates a rate for repair of the damage. The estimate for repair is slow, with a half-life of approximately 33 h in the case of PCBZ in *H. azteca*. This rate for damage repair contrasts with expected rapid reversibility of nonpolar narcosis as set forth in the CBR model [1]. Thus, even relatively large changes in water concentration as observed in the experiments between water renewals have little impact as long as both the elimination and the damage recovery rate are relatively slow.

The time dependence for the body residue required for 50% mortality is very steep for short exposure durations and comes to an apparent asymptote after about 100 h. If the classic approach to toxicity testing is used, no statistical difference would be found for the body residue required for 96- and 600-h exposures. Thus, assessors would assume that no temporal effect existed on body residues required to produce a toxic response in this species with PCBZ. This relatively constant body residue likely comes from the relatively short half-life for repair for PCBZ (33 h). This short half-life for repair is consistent with a mechanism of reversible narcosis. The need for determining the time dependence lies in the need for improved interpretation of response to a range of exposure conditions that likely occur in the field so that risk assessments can be improved. For instance, the model permits prediction of the time required for the organism to completely recover between stochastic events. Assuming that five half-lives of repair are required for full recovery, then 160 h between exposures are required to assume that the organism is not carrying any residual damage after elimination is complete. Thus, in the case of pulsed exposures from stochastic events, some residual damage would be expected if the time between events is less than 160 h. These insights cannot be obtained from the traditional experimental designs that do not develop a full range of time-dependent toxicity data.

Growth rate has been established as a sensitive endpoint for evaluating the toxicity of sediments using *H. azteca* [20]. Further, it is observed that the response to sublethal endpoints

can produce more sensitive measures of response based on the body residue required to produce mortality [21,22]. The growth rate determined for *H. azteca* in a 28-d experiment was found to be similar in sensitivity to mortality largely because of the variability in the body residue associated with reduction in growth (Fig. 8). This was thought to be partly a result of the difficulty in weighing individual organisms and measuring the body residue in individual organisms. However, part of the variability is a result of the differences in growth rate among individuals. The procedural difficulties could be overcome by determining the concentration and weights of groups of individuals instead of individual animals. One of the objectives was to demonstrate that the time-dependent toxicity on a body residue basis was not due to biotransformation creating a toxic metabolite. The fit of the PCBZ temporal data to the damage assessment model exhibited both similarities and differences compared to the response of *H. azteca* to PAH congeners [5]. In general, PCBZ was about four times more potent than pyrene, a compound of similar log K_{ow}. However, the damage repair rate for the two compounds was similar with half-lives for repair that were only a factor of two different (33 h for PCBZ and 69 h for pyrene [5]). It seems reasonable that the repair process should require similar times if the mechanism of damage repair is similar. Since both compounds are expected to act by nonpolar narcosis, the repair process should be similar. Overall, this comparison suggests that should *H. azteca* be exposed in mixture, direct molar additivity will not account for the response, but a toxic unit approach with additivity might.

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